The effect of anaerobic atmospheres on the stability of the virulence-related characteristics in *Yersinia enterocolitica*

This report presents information on the stability of the virulence plasmid in Yersinia enterocolitica *under different anaerobic atmospheres. Exposure of plasmid-bearing virulent cells both in growing and stationary phases to anaerobic atmospheres consisting of 80% N_2 plus 20% H_2; 94% CO_2 plus 6% H_2 or under vacuum for 24 h at 28°C did not lead to the loss of the virulence plasmid from these cells. Virulence assays using crystal violet binding, low-calcium response, Congo red uptake, and hydrophobicity by latex particle agglutination, indicated that the cells were still virulent.*

The association of human illness with the consumption of food contaminated with *Yersinia enterocolitica* is well documented (Doyle and Cliver 1990, Lee et al. 1990, Kapperud 1991). Since Yersiniae can grow at low temperatures, refrigerated foods are potential vehicles for the growth of the organisms (Gill and Reichel 1989, Doyle and Cliver 1990, Kapperud 1991). Strains of all serotypes implicated in human disease harbor a plasmid of molecular weight of 40 to 45 MDa which is directly involved in the virulence of this bacterium (Zink et al. 1980, Portnoy and Martinez 1985, Kapperud 1991). A number of temperature dependent phenotypic characteristics associated with the virulence plasmid have been described and used to detect plasmid-bearing virulent strains of *Y. enterocolitica* (Portnoy and Martinez 1985, Bhaduri et al. 1987, Robins-Browne et al. 1989, Doyle and Cliver 1990, Bhaduri et al. 1991, Kapperud 1991, Kwaga and Iversen 1991). Virulent *Yersinia* strains dissociate into virulent and avirulent clones after cultivation. Such dissociation is associated with plasmid loss, an event which is facilitated by culturing at 37°C (Zink et al. 1980, Portnoy and Martinez 1985, Bhaduri et al. 1987, 1991) or by extended storage at 4°C (unpublished data). Consequently, loss of this plasmid results in the loss of virulence and the concomitant disappearance of associated phenotypic characteristics. Recently, we studied the stability of the virulence plasmid in *Y. enterocolitica* at elevated temperatures and at various sodium chloride concentrations and acidic pH by using the plasmid-associated crystal violet (CV) binding property to detect the presence of the plasmid in the cells and as an indicator of virulence (Bhaduri et al. 1988, Bhaduri and Mertz 1989). Although some literature is available on the growth and survival of *Y. enterocolitica* under anaerobic atmospheres in meat packages and milk (Ahmad and Marchello 1989, Gill and Reichel 1989,

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Kleinlein and Untermann 1990, Rowe 1988), no information is available about the stability of the resident plasmid under these conditions. Due to the unstable nature of the plasmid (Zink et al. 1980, Portnoy and Martinez 1985, Bhaduri et al. 1987, 1991), the possibility of plasmid loss in various anaerobic conditions found in food processing cannot be excluded. The present study was initiated to determine whether similar anaerobic atmospheres found in food processing trigger the loss of the virulence plasmid in *Y. enterocolitica* by using CV binding (Bhaduri et al. 1987), low-calcium response (Lcr) (Bhaduri et al. 1991), Congo red (CR) uptake (Bhaduri et al. 1991) and hydrophobicity by latex particle agglutination (LPA) test (Bhaduri et al. 1987, 1991) which depend on the presence of virulence plasmid.

Recent reports indicate the emergence of Serotype O:3 of *Y. enterocolitica* as the major cause of gastroenteritis in the United States (Lee et al. 1990, 1991, Metchock et al. 1991); therefore, plasmid-bearing (P^+) and its isogenic plasmidless (P^-) strains GER (Serotype O:3) of *Y. enterocolitica* were used in this study. A detailed description of the strains, source and preparation of inocula, and incubation conditions are given elsewhere (Bhaduri et al. 1987, 1991). To study the effect of atmospheric conditions on the stability of the virulence plasmid, P^+ cells were grown in the following two ways. First cultures of P^+ cells were allowed to grow in brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI) for 18 h at 28°C under anaerobic conditions as described below to concentration of 10^9 cfu ml^{-1} to determine if the plasmid was lost during the growing phase. Second, cultures of P^+ were first grown to stationary phase (10^{10} cfu ml^{-1}) in BHI broth for 24 h at 28°C aerobically with shaking and then placed in the appropriate atmospheric conditions as described below and incubated at 28°C for 18 h to determine the loss of plasmid. The following anaerobic atmospheric conditions were used: (1) 80% N_2 plus 20% H_2 gas atmosphere (GasPak anaerobic system from Becton Dickinson Microbiology Systems (BBL), Cockeysville, MD); (2) 94% CO_2 plus 6% H_2 gas atmosphere flushed three times and then filled with the same gas; and (3) under vacuum. GasPak jars were for gas atmospheres. Oxoid anaerobic jar (Oxoid USA, Inc., Columbia, OH) and vacuum pump (Gast Air Pump, model 1033-S; Gast Manufacturing Corporation, Carlstadt, NJ) were used for the vacuum treatment. Maintenance of anaerobic conditions was monitored by disposable anaerobic indicator (BBL). The cells were diluted to 10^3 cells ml^{-1} and surface plated onto BHI agar and CR-BHI agarose (Bhaduri et al. 1987, 1991). Plates were incubated at 37°C for 24 h. Plates were then counted and the presence of the virulence plasmid in the cells was detected by CV binding, Lcr, CR uptake, and hydrophobicity by LPA test (Bhaduri et al. 1987, 1991). P^+ clones were enumerated by using the first three techniques. P^+ and P^- cells grown aerobically in BHI broth for 18 h at 28°C were used as positive and negative controls for the confirmation of plasmid associated virulence tests used in this study.

The data in Table 1 show that under anaerobic conditions, there was no effect on the overall number of viable cells. More than 94% of cells retained the resident plasmid. Similarly the numbers of viable cells from the stationary phase remained unchanged under all three anaerobic conditions and more than 96% of surviving cells again retained the resident plasmid (Table 2). At both growing and stationary phases the presence of the virulence plasmid in the cells was

Table 1. Effect of atmospheric anaerobic conditions on the stability of virulence plasmid in growing phase.

Anaerobic growth conditions	Total number of colonies on BHA	No. (%) of colonies bound to CV	Total number of colonies on CR-BHO	No. (%) of colonies showing Lcr and CR uptake
N_2+H_2	40	39(97)	44	43(98)
CO_2+H_2	39	36(92)	47	41(89)
Vacuum	40	38(95)	51	48(94)
Control O_2 (P^+)	48	44(91)	69	67(97)
Control O_2 (P^-)	45	0	43	0

P^+ cells were grown for 24 h at 28°C in BHI broth under anaerobic atmospheric conditions. The cells were diluted to a concentration of 10^3 cells per ml determined by A_{600} in BHI broth. The cells were then surface plated on BHA and CR-BHO and incubated at 37°C for 24 h. Total cells were counted and the presence of the plasmid in the cells were detected by crystal violet binding (appearance of dark violet colonies), low-calcium response (appearance of pinpoint colonies), Congo red uptake (appearance of red pinpoint colonies). The P^+ and P^- cells grown aerobically (O_2) were tested as control. BHA, brain heart infusion agar; CR-BHO, Congo red brain heat infusion agarose; CV, crystal violet; Lcr, low-calcium response; CR, Congo red.

also confirmed by hydrophobicity by latex particle agglutination test under different anaerobic conditions as described in Table 1 and 2 (data not shown). In general the percentage (4–6%) of converted P^+ cells to P^- cells in anaerobic conditions did not alter significantly from the converted P^+ cells to P^- cells which were grown aerobically as a control. The data presented here are the results of one of several experiments that all showed similar responses. These results indicate that when P^+ virulent cells of *Y. enterocolitica* are exposed to anaerobic atmospheres during growth or at the stationary phases, most of the cells retain the resident plasmid and presumably are virulent. Of greater significance is the observation that cells of *Y. enterocolitica* held under anaerobic situations retain the virulence plasmid and are still potentially capable of causing food-poisoning under the appropriate conditions.

Table 2. Effect of anaerobic atmospheric conditions on the stability of plasmid at stationary phase.

Anaerobic growth conditions	Total number of colonies on BHA	No. (%) of colonies bound to CV	Total number of colonies on CR-BHO	No. (%) of colonies showing Lcr and CR uptake
N_2+H_2	56	55(98)	57	55(96)
CO_2+H_2	44	43(97)	48	46(95)
Vacuum	42	41(97)	68	66(97)
Control O_2 (P^+)	52	52(100)	53	53(100)
Control O_2 (P^-)	48	0	44	0

P^+ cells were grown aerobically for 18 h at 28°C in BHI broth. The cells were then placed in atmospheric conditions and incubated at 28°C for 24 h. The cells were diluted to a concentration of 10^3 cells per ml determined by A_{600} in BHI broth. The cells were then surface plated on BHA and CR-BHO and incubated at 37°C for 24 h. Total cells were counted and the presence of the plasmid in the cells were detected by crystal violet binding (appearance of dark violet colonies), low-calcium response (appearance of pinpoint colonies), Congo red uptake (appearance of red pinpoint colonies). The P^+ and P^- cells grown aerobically (O_2) were tested as control. BHA, brain heart infusion agar; CR-BHO, Congo red brain heat infusion agarose; CV, crystal violet; Lcr, low-calcium response; CR, Congo red.

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